EFFECTIVENESS OF OZONATED WATER IRRIGATION AGAINST AN ESTABLISHED *ENTEROCOCCUS FAECALIS* BIOFILM IN ROOT CANAL TREATED TEETH *IN VITRO*

By

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INTRODUCTION

The role of microorganisms in pulpal and periapical infections is well known. Over 50 years ago, the presence of a microbiota was found to be a major determinant in the development of pulpal and periapical disease (1). Thus, one of the primary goals in root canal therapy is to lower the microbial load as much as possible. This goal is accomplished through the use of mechanical instrumentation (2), chemical irrigants (3, 4), and intracanal medicaments (5-7). Since it has been shown that mechanical instrumentation alone cannot clean 100% of the root canal system (RCS), including isthmuses, anastomoses, fins, and deep within dentinal tubules (8), extensive, ongoing research has been devoted to developing an ideal endodontic irrigant that is both an effective antimicrobial and is non-irritating to periapical tissues and viable human cells in order to clean these intricacies of the RCS.

Today, sodium hypochlorite (NaOCl) serves as the endodontic irrigant of choice due to its nonspecific, potent, antimicrobial efficacy, ability to dissolve organic tissue, ease of use, and low cost (4, 9). NaOCl works through the formation of chloramine ions that interfere with cellular metabolism, cause irreversible enzymatic inactivation of bacteria, as well as lipid and fatty acid degradation (10). While various concentrations exist, most dental practitioners in the United States use NaOCl with a concentration around 6% for their standard, nonsurgical root canal procedures due to the fact that the higher concentration makes it more effective against microbes and enhances tissue solvent action inside the RCS (3, 11). Unfortunately, the major drawback of NaOCl is its toxicity to human cells making it unsafe to use at the higher concentrations, including the traditional 6%. In clinical situations, such as resorptive cases and in teeth with immature, open apices, lower concentrations around 1.5% have been used (12-14). In these

resorptive and regenerative cases, it has been indicated to use gentle irrigation with a lower concentration of NaOCl, or an alternative irrigant altogether (15-17). This is suggested in order to reduce the chance of clinical extrusion and a possible NaOCl accident and to prevent the killing of any live human cells that may have direct contact or interaction with the RCS; such as the stem cells of the apical papilla which are critical cells involved in regenerative endodontic procedures (REPs) (17).

As previously mentioned, ongoing research has been dedicated to several other methods of endodontic disinfection in order to reduce the risk of adverse reactions and toxicity to human cells that can be seen with NaOCl irrigation. Some of these other disinfection methods include photodynamic therapy, chlorhexidine, diode lasers, electromagnetic stimulation, and ozone (16, 18-26). The present study will compare the antimicrobial activity of various concentrations of ozonated water to two of the more commonly used concentrations of NaOCl (6% and 1.5%) against an established biofilm of *Enterococcus faecalis* formed inside the root canals of extracted human teeth.

Ozone, a naturally occurring molecule consisting of three oxygen atoms, has been used as a powerful disinfectant for over 150 years in a number of different industries. The first application of ozone in the medical field was to treat gaseous, post-traumatic gangrene in the First World War (27). Since then, technological advances, including the invention of the medical grade ozone generator, have allowed for significant progress in the field of ozone therapy and use. Today, ozone is used for wastewater treatment in a number of cities throughout the world, in food processing and storage, and is beginning to gain more application in the medical and dental fields for its powerful disinfecting properties. Ozone is one of the most powerful oxidants known to man that kills bacterial

cells by oxidizing the lipid and lipoprotein components of their cell membranes rendering them nonviable (28). This action is *specific* and selective to microbial cells (28). Nagayoshi et al investigated the efficacy of ozone on the survival and permeability of oral microorganisms and found that ozonated water strongly inhibited the accumulation of oral microbiota and when applied with sonication, had nearly the same antimicrobial effect as 2.5% NaOCl (29, 30). Numerous other studies have found various forms and concentrations of ozone to be effective against several different oral microorganisms including but not limited to *E. faecalis, S. mutans, S. sobrinus, S. sanguis, P. gingivalis, and A. actinomycetemcomitans* (24-26, 30).

Potential concerns regarding the use of ozone as an endodontic irrigant include its effect on human cells, its cost and ability to produce, and its duration of action once created. Much research has been dedicated to determining the biocompatibility of ozone on human cells. A high level of biocompatibility of aqueous ozone has been established against human epithelial cells, gingival fibroblasts, and periodontal cells (30-32). Topical application of ozone is not toxic for human cells and has actually been shown to promote wound healing by increasing oxygen tension, increasing granulation tissue formation, and increasing the expression of transforming growth factor beta (TGF-B) and vascular endothelial growth factor (VEGF)(32). Millar et al investigated the safety of two commercially available ozone delivery devices used in dentistry (Ozi-Cure and HealOzone) and found that when used with adequate nearby suction, the level of ambient ozone was reduced to zero and therefore considered safe to use (33). Once ozonated water is produced, it must be used within a reasonable timeframe due to the fact that the ozone will dissociate into oxygen molecules and therefore deplete the water's

concentration of ozone. A study in Spain working with ozonated saline, found that after five minutes the concentration of ozone decreased by 17%, after ten minutes it decreased 29%, after fifteen minutes by 37%, and after twenty minutes it decreased by 43% (34). As a result, it is crucial to use the ozonated solution immediately or shortly after it is produced in order to achieve maximum results. In addition, any and all containers or syringes ozonated water will be stored in must be made of glass, silicon, or Teflon as ozone will degrade plastic materials due to its high oxidative power. These few drawbacks may slightly increase costs for dental practitioners should they choose to use ozone as part of their armamentarium. However, if ozone can be proven to be an effective, biocompatible antimicrobial without producing harmful decomposition products, it should garner consideration in certain clinical situations.

Previous research has shown that most oral and endodontic microbes do not live as simply free-floating bacteria, but rather in a complex, interconnected superstructure known as a biofilm (35, 36). Studies have shown that these biofilms can be up to 1000 times more resistant to antibacterial agents (35, 36). Due to its presence and association in many endodontic infections (37-39), an *E. faecalis* biofilm will act as this project's model to evaluate the antimicrobial effectiveness of ozonated water. Estrela *et al* demonstrated how *E. faecalis* serves as a model to study antimicrobial strategies in endodontics due to it consistently adhering to collagen structure, colonizing dentin surfaces and tubules, and progressing to form a biofilm (40). Duarte *et al* developed an effective protocol to assess anti-biofilm activity against an established *E. faecalis* biofilm in single-rooted teeth that will be used in this experiment (41).

The use of ozonated water in endodontics may prove to be of value in certain clinical cases. With its proven antimicrobial effects and high biocompatibility, ozonated water could be considered as an alternative or an adjunctive endodontic irrigant to the more traditional and potentially toxic NaOCl, especially in cases dealing with resorptive defects or immature teeth with open apices. This study will further evaluate the antibacterial effectiveness of two different concentrations of ozonated water against a common endodontic bacterial biofilm and help add to the body of knowledge regarding the use of ozone in dentistry and its potential clinical implications.

OBJECTIVE

- The aim of the current study will be to evaluate the anti-biofilm activity of different concentrations of ozonated water compared to various concentrations of sodium hypochlorite against an established endodontic biofilm of *E. faecalis* formed inside the root canals of extracted human teeth.

HYPOTHESES

- *Null:* Endodontically treated teeth irrigated with ozonated water will not demonstrate a statistically significant decrease in the *E. faecalis* biofilm compared to those treated with sodium hypochlorite
- *Alternative:* Endodontically treated teeth irrigated with ozonated water will demonstrate a statistically significant decrease in the *E. faecalis* biofilm compared to those treated with sodium hypochlorite

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Evidence of dental pain and treatment can be found dating all the way back to 5000 BC, where a description of a "tooth worm" is inscribed on an ancient Sumerian text as the cause for dental decay and pain (42). These "tooth worms" boring into teeth, wiggling around, and gnawing on tooth structure were thought to be the cause of dental pain for centuries among various cultures and dynasties throughout the world (43). Around 500-300 BC, Aristotle and Hippocrates began to write about dentistry, including about the eruption pattern of teeth and various dental procedures like treating caries and gum disease, as well as extracting teeth with forceps, and using wires to stabilize teeth and broken jaws (42, 43).

The beginning of dentistry as a profession can trace its origin back to 1687, when Charles Allen was credited with writing the first book in English dedicated exclusively to dentistry (44). It was around this time, based on findings from Anton von Leeuwenhoek, the "father of modern microscopy," that the "tooth worm" theory was beginning to be questioned (44). In 1700, von Leeuwenhoek wrote a letter to the Royal Society of London explaining that the "tooth worms" from decayed teeth were the same as the "cheese worms" found in cheese sold at a cheese shop (43). Twenty-eight years later, in 1728, Pierre Fauchard, the "father of modern dentistry," released his book *The Surgeon Dentist*. In it, Fauchard accurately described the contents of the dental pulp which led to the dispelling of the "tooth worm" theory and led to the "Empirical Era" of dentistry (44). Fauchard also explained the practice of opening teeth to relieve dental abscesses and drain pus as well as how to extirpate the dental pulp using a small pin (44). The birth of modern-day endodontics as we know it had begun.

In 1756, a German dentist named Phillip Pfaff, described one of the first recorded pulp-capping procedures in which he placed a concave piece of gold or lead over the exposed pulp so that the metal was not touching the nerve (45). The first recorded technique of an endodontic procedure occurring in North America was written by Robert Woofendale, who came to New York from England in 1766. Woofendale's technique was aimed at alleviating pain and involved cauterizing the dental pulp with a hot instrument and packing cotton into the canals of the tooth (46). Woofendale went on to publish the book, *Practical Observations on the Human Teeth*, in which he described his techniques to alleviate pain caused by exposed nerves in teeth. In these situations, Woofendale used the application of oil from cinnamon, cloves, turpentine, or any chemical oil for pain relief; he also noted that if repeated for some time, this will often destroy the nerve (46). By the end of the 18th century, another German dentist, Frederick Hirsch, discovered and wrote about how tapping on a tooth can help identify and diagnose occult dental disease. Hirsch found that a diseased tooth elicited pain on percussion (47).

The next phase in the development of modern-day endodontics was termed the "Vitalistic Era" and persisted for close to 70 years. In 1805, J.B. Gariot was one of the first people to identify a connection between pulpal treatment and the vitality of a tooth (48). Gariot believed that the destruction of the dental pulp does not devitalize the tooth (48). Leonard Koecker, author of *Principles of Dental Surgery* and an early pioneer of the "vitalistic" theory, believed that when the dental pulp was destroyed, the entire dentinal core of the tooth died. As a result, the tooth was thought to became a foreign body that necessitated extraction in order to avoid inflammation and possible necrosis of

the vital tissues surrounding it (49). In an effort to avoid loss of the tooth, Koecker popularized a pulp capping procedure, very similar to that described by Pfaff a century earlier, that dominated pulp treatment procedures for close to 50 years (48). In 1829, the "vitalistic" theory was indoctrinated by S.S. Fitch in his book, *System of Dental Surgery.* Fitch believed that teeth were structured similar to hollow bones with an inner periosteum supplying the blood supply and nutrients to the crown of the tooth and inner root surface and an outer periosteum (the periodontal membrane) supplying nutrients to the outer root surface. According to this theory, when the pulp was removed, only the crown of the tooth lost its vitality entirely as the roots maintained nourishment from the periodontal membrane (48). This led to the practice of removing the crowns of teeth after pulp extirpation and the placement of pivot crowns on the remaining root(s) (50). On the other side of the endodontic aisle at the time were the "non-vitalists." This group included Hunter, Cuvier, and Robertson from England who believed that dentin had no circulation, sensibility, capability of repair, or any of the properties of living tissue (48).

Up until 1836, all vital pulp therapy would have been extremely painful as no sedative material or local anesthetic had been discovered yet. Shearjashub Spooner from New York experimented with and found that arsenic applied to pulps of teeth would devitalize the nerve prior to extirpation making the procedure painless (44). This discovery however led to imprudent overuse of arsenic in dentistry to treat hypersensitive dentin and symptomatic pulps. Leakage of the arsenic through the root canals destroyed adjacent vital supporting structures and the periodontium (44).

The first endodontic file, similar to those used today, was invented by Edwin Maynard in 1838 by filing a watch spring. This technical innovation allowed

practitioners to enter into and clean smaller canals like those found in premolars and molars. In 1839, Baker used instruments like those developed by Maynard to develop a protocol for the treatment of an exposed nerve. Baker wrote in the *American Journal of Dental Science* that the treatment for an exposed nerve was to remove the nerve, clean the canal, and fill the canal with gold foil (44). In doing so, Baker is credited with writing the first published account of endodontic therapy from pulpal extirpation, to canal cleaning, and finishing with canal obturation.

Many other technological advancements were made in the field of endodontics in the 19th century. The use of a rubber dam was introduced by Barnum, gutta percha was suggested as a root filling material by Bowman, an antiseptic technique created by Lister was transitioned to pulpal treatment, and the first electric pulp test was introduced into endodontics by Magitot in 1867 (44, 47, 51, 52).

In 1878, G.O. Rogers suggested that pathogenic microorganisms might be the most common cause for pulpal disease and that successful treatment required the elimination or destruction of these organisms (48). This conclusion based on the recognition of the pathogenicity of bacteria, led to the demise of the "vitalistic" theory and to the birth of the new "septic theory." A few years later, Arthur Underwood further developed this new theory. Underwood proposed that the suppuration of the pulp and the associated abscess, depends on the toxic effects of the pathogenic microorganisms (48). Underwood believed that if the pathogens were eliminated using powerful antiseptics, the disease could be alleviated. As long as the contents of the pulp chamber and canal were sterilized, whether or not the pulp was vital made no difference. This concept provided clinicians a new basis for pulp therapy and various caustic antiseptics were used in

endodontic treatment over the next 30 years (52). Some of these agents included arsenic, formalin, sodium dioxide, chlorophenol, sulfuric acid, paraformaldehyde, formocrescol, and glycerol with hydrochloric acid (44, 47, 51, 52).

Throughout the 19th century, many materials were experimented with and used to obturate root canals. As mentioned, Baker advocated the use of gold foil to pack into canals as early as 1839 (44). Towards the late 1800s, Bowman introduced a solution of chloroform and gutta percha, termed chloropercha, used with gutta percha cones to obturate root canals (52). This material was quickly accepted by many dentists, including its greatest advocate M.L. Rhein, who further developed and described its technique a decade later. In 1884, Cassius Richmond began teaching an obturation method that involved sterilizing the root canal with phenol and iodine, sealing the foramen with a sterile, solid material, and filling the remaining canal with an aseptic cement (52). Richmond advocated using carbolized orangewood as a root canal filling material. In 1890, C.T. Gramm, from Chicago, was using copper points to obturate root canals (52). By 1911, Callahan introduced a new technique using rosin and chloroform to fill root canals and asserted that this technique provided a means to penetrate and seal dentinal tubules to provide a better hermetic seal (53, 54).

Another important aspect of dental and endodontic treatment, anesthesia, was beginning to make marked advances in the 19th century. Koller introduced cocaine as a topical anesthetic in the 1880s. In 1890, E.C. Briggs was using cocaine topically to anesthetize dental pulps (51). That same year, Funk improved the procedure by forcing a solution of cocaine directly into the pulp chamber and applying pressure to anesthetize the pulp (47). While the use of cocaine as an anesthetic agent persisted for around 20

years, many dentists used it sparingly or not at all due to its toxicity (55). In 1905, Einhorn developed procaine (Novocaine) but its technique was tedious and cumbersome to use (56). H.S. Vaughn, from New York, is the first person credited with using infiltration anesthesia prior to pulpal extirpation (57). Nearly a quarter century later, block anesthesia techniques were perfected (52).

In 1895, a monumental breakthrough in medicine and dentistry occurred when Wilhem C. Roentgen accidentally discovered X-rays (52). While testing if cathode rays could pass through glass, Roentgen found that mysterious light could pass through most substances and leave shadows of solid objects. Since he did not know what the rays were, he called them "X," for unknown, rays (58). Shortly after this discovery, W. J. Morton took the first dental radiograph in America and C. Edmund Wells began to routinely use X-rays in his dental practice in New Orleans (52). For the first time, dentists were able to visualize the results of their root canal procedures. The use of X-rays was adopted by the American dental profession around 1910 as a diagnostic tool that revealed previously unknown pathologic conditions in the oral cavity (59).

By the turn of the century, trends in dental thought and teachings began to change. Evidence of this occurred in 1904, when Frank Billings directed the attention of dentistry and medicine to the supposed relationship between oral infection and systemic disease, specifically bacterial endocarditis (56). In 1909, E.C. Rosenow, a student of Frank Billings, published a study concerning the bacteriologic aspect of root canal therapy. Rosenow showed that streptococci were present in many diseased organs and were able to spread through the bloodstream and infect other distant sites (56). That same year, Mayrhofer published a work linking pulpal infection to specific microorganisms. He

found streptococci to be involved in approximately 96% of the cases studied (60). These findings led to the association between oral sepsis and systemic disease, the foundation for what would become known as the "focal infection theory." A pioneer of this unfortunate theory was William Hunter, an English physician and pathologist. Hunter lectured on focal infection at McGill University in Montreal, Canada and this lecture was later published in a respected medical journal of the time, the *Lancet* (53). This type of thinking resonated within the medical and spawned a large following of American dentists for over 25 years, leading to unnecessary, widespread edentulism for countless unfortunate patients in the name of systemic disease treatment and prevention.

Fortunately for dentistry and the field of endodontics in particular, a few individuals resisted prescribing to this theory. This group included men like Coolidge, Johnson, Rhein, Callahan, Grove, Prinz, and others. It was through their effort and perseverance, relying on the safe and effective local anesthetics that had been developed, X-rays as a new and reliable diagnostic aid, and further developing aseptic techniques with bacteriological and histological methods, that the principle of preserving the pulpless tooth was able to survive (51, 61).

Throughout the early 20th century, careful attention and much research was spent on developing more biocompatible materials to use in endodontic therapy. Hermann began to question the safety of many nonbiological substances being used such as phenol, paraformaldehyde, camphor, and other materials foreign to the body. In 1920, he began to use a calcium hydroxide mixture to fill root canals. Ten years later, this same material was being used for pulp capping, pulpotomies, pulpectomies, and in the treatment of infected root canals (62). In 1925, Rickert proposed the use of a cementing medium, or

sealer, in conjunction with a gutta percha cone for obturating root canals. The sealer was able to be expressed laterally and apically through downward pressure of the cone and obturate any potential accessory or lateral canals and therefore provide a better overall seal. This technique would later improve through the creation of lateral condensation (56). In 1931, Rickert and Dixon began a series of experiments that would form the basis for the hypothesis of the "hollow tube effect." This suggests that if voids are present in a root canal filling, the space can fill with tissue fluids and ultimately leak to the periapical tissues and result in inflammation (63). In 1933, E. A. Jasper introduced silver points to dentistry. These points were standardized to have the same diameter and taper as the instruments used to clean and shape the root canals.

The eventual demise of the focal infection era came with the publication of three works in 1937. First, Logan showed that the presence of microorganisms in tissues did not necessarily imply the presence of infection. Logan's work demonstrated that bacteria can be and often are present in normal tissues without any pathological significance (46). Next, Tunnicliff and Hammond found bacteria in the pulps of extracted teeth without any evidence of inflammatory tissue changes (56). Finally, Cecil of Cornell Medical College, reported 200 cases of arthritis in which treatment consisted of extraction of suspected teeth. He found little benefit from surgical intervention and this finding led to the conclusion that any clinical improvement after extraction was likely a mere coincidence (64). These works, among others, provided scientific evidence based on sound histological, biological, and pathological findings and were able to usher in a new era in root canal therapy: "the scientific era." During this era, sound laboratory research and

abundant clinical evidence proved that a pulpless tooth did not play a role in the development or persistence of systemic disease (65).

In 1943, organized endodontics began when a group of 20 men met in Chicago and formed the American Association of Endodontists (AAE) (53, 61). Harry Johnston is credited for coining the term "endodontia," which comes from the Greek words: "endon" (within) and "ho dontas" (a tooth). Three years later, in 1946, the first dental journal dedicated entirely to the subject of endodontics, *The Journal of Endodontics*, was published. By 1963, over 200 American dentists were limiting their dental practice to endodontics (61). Due to the field's extraordinary growth over the next 25 years, along with the tireless efforts by leaders of the AAE, the American Dental Association recognized endodontics as a dental specialty in 1963 (66).

Since the foundation of the AAE and the recognition of endodontics as a dental specialty, the field has erupted with scientific and technological breakthroughs. Through the development and implementation of things such as the dental operating microscope, new and improved nickel titanium rotary instruments, adjunct irrigation techniques and devices, CBCT imaging, and ongoing research and clinical studies in regenerative endodontics, the root canal procedure is becoming quicker, more predictable, and less painful for everyone involved.

THEORY OF ENDODONTICS

Just after the field of endodontics was officially recognized as a distinct dental specialty, a monumental study by Kakehashi, Stanley, and Fitzgerald in 1965 revealed

the relationship between microorganisms and the development of endodontic and periapical pathosis (1). Their study involved exposing the pulps of teeth in two groups of rats: gnotobiotic (germ-free) and conventional. Their findings showed that the pulps in the germ-free group remained vital whereas the conventional rats experienced pulpal necrosis and periapical pathology (1). From this, the authors were able to conclude that bacteria were the true cause for pulpal and periapical disease.

The results of this study led others to investigate how and why pulpal necrosis and periapical inflammation can occur. In 1974, Bergenholtz found microorganisms inside previously traumatized teeth exhibiting periapical destruction (67). In 1981, Moller et al discovered that microbial invasion of necrotic pulps was required for subsequent periapical inflammation and apical periodontitis to develop and that necrotic pulps alone without bacteria would not lead to endodontic disease (68). These studies, among others, laid the foundation for understanding how and why endodontic disease develops. Bacteria from the oral cavity invade the dental pulp by way of caries, restorative work, and/or trauma. The presence of bacteria inside the pulp leads to inflammation and, if left untreated, possibly pulpal necrosis (69).

Once it was established and accepted that bacteria were the cause for endodontic disease and apical pathology, research was devoted to finding out how bacteria played that role. Upon initial invasion of the pulp, bacterial virulence factors such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) in gram-negative and gram-positive bacteria, respectively, result in inflammatory cytokines and neuropeptides to be released (70-72). These virulence factors and the inflammatory mediators result in pulpal inflammation and symptoms of pulpitis (73, 74). If the inflammation is left untreated and

persists, micro-abscesses can form in the dental pulp leading to pulpal necrosis (75). A necrotic pulp loses its innate immunological defense systems and therefore, more bacteria can easily invade through exposed dentinal tubules and thrive in the necrotic root canal system (70, 76). As these microorganisms and their associated virulence factors spread throughout the root canal system, they eventually reach the apical tissues and lead to periapical inflammation and apical periodontitis (77).

The countless studies that identified bacteria as the cause for pulpal inflammation and endodontic disease and the research that discovered the various mechanisms of the microorganisms helped lay the foundation on which endodontic treatment is based (1, 67-73). The primary goal of endodontics is to reduce the pathogens and their associated toxins inside the root canal system to an acceptable level for the body to initiate the healing process (78). There is a direct correlation between the adequate reduction of the insulting microbial load associated with the root canal system and endodontic success (79).

One of the pioneers to evaluate the importance of microbial reduction in endodontic therapy was Dr. G.G. Stewart. In 1955, he conducted a study that laid out the three critical phases for endodontic treatment success: chemomechanical preparation, microbial control, and obturation of the root canal (79). Stewart considered chemomechanical preparation of the root canal system the most important phase of endodontic treatment crucial for success for a number of reasons: as the root canal is enlarged, the number of microorganisms and debris in which these pathogens can survive is reduced, as the internal diameter of the canal is increased, the easier it is for

disinfecting irrigants and medicaments to reach all aspects of the root canal system and the simpler it is to properly obturate the canal(s) (79).

Taking the results of Stewart's study and expounding on his three pillars for successful root canal therapy, Grossman laid out thirteen principles he found endodontic treatment relied upon for success (80):

1. Aseptic technique.
2. Keep instruments within the root canal system.
3. Never force instruments apically.
4. Canal space must be enlarged to facilitate irrigation and obturation.
5. Continuous irrigation with an antiseptic.
6. Solutions and medicaments should remain within the root canal.
7. Fistulas do not require special treatment.
8. A negative culture should be obtained before obturation of the root canal.
9. A hermetic seal of the root canal system should be obtained.
10. Obturation material should not be irritating to the periapical tissues.
11. Proper drainage should be established in acute abscesses.
12. Injections directly into infections should be avoided.
13. Apical surgery may be required to facilitate healing of the pulpless tooth.

These principles would go on to guide the way endodontic procedures were being completed and help lay the basis for successful root canal therapy for years to come.

One of the first practitioners to illuminate the importance of obturating the root canal system and creating a properly sealed canal was Schilder in 1967. Schilder emphasized the biologic necessity of eliminating bacteria and their toxic by-products

from within the root canal system for endodontic therapy to be successful but pointed out that prognosis for an endodontically treated tooth is improved if the root canal system is completely obturated and sealed from both the oral cavity and the periapical tissues (81). Schilder created a technique using heated pellets of gutta percha inserted into and condensed inside the root canal in order to create a three-dimensional fill and a hermetic seal. This technique revolutionized the way root canals were filled and drastically increased endodontic treatment success (82).

MECHANICAL INSTRUMENTATION

Instrumentation/preparation of the root canal system serves both biological and mechanical purposes during root canal therapy. Biologically, instrumentation breaks up microbial biolfilms, reduces the microbial load and their associated toxins, and helps remove organic tissue and debris from inside the root canal (83). Mechanically, instrumentation enlarges the root canal space and ensures adequate taper to help facilitate the delivery of powerful disinfectants and ultimately three-dimensional obturation of the entire root canal system (81, 84).

Bacteria have been shown to invade dentinal tubules up to 300 microns, therefore, it is critical to remove not only the necrotic tissue and microbes associated within the root canal proper, but also the infected dentin along the root canal walls (85). In accomplishing this task, it is important to maintain all endodontic instruments within the root canal system and to keep the natural shape and curvature of the canals in order to avoid iatrogenic errors like ledging, zipping, or perforating the root canal which could

result in damage to the periodontal tissues or incomplete disinfection of the root canal system (86-88).

While it has been shown that mechanical instrumentation can reduce the microbial load anywhere from 100- to 1000-fold inside the root canal system (2), the anatomical complexities including fins, anastomoses, apical deltas, lateral and accessory canals, inhibit current endodontic files from reaching 100% of the root canal system (8, 89). For these reasons, an additional step in endodontic therapy is necessary to ensure adequate disinfection: chemical irrigation.

CHEMICAL IRRIGATION

Irrigation is a key step in successful root canal therapy and serves several functions depending on the irrigant being used. Irrigants act as a lubricant inside the canal and reduce the friction between the instrument and dentin, improve the cutting ability of files, dissolve tissue and debris, help reduce heat generated inside the canal, and, perhaps most importantly, have a washing effect along with antimicrobial and antibiofilm properties (90). The ideal endodontic irrigant would be an effective germicide/fungicide, be non-irritating to periapical tissues, have a sustainable antimicrobial effect, remain active in the presence of bodily fluids, have a low surface tension, be non-staining to tooth structure, unable to initiate an immune response from the host, able to remove both organic and inorganic debris and disinfect dentinal tubules, have no adverse effect on physical properties of the tooth or obturation materials, be easy to use, and be relatively inexpensive (69). Presently, there is no single irrigant that

possesses all of these characteristics; thus, a variety of irrigants are used throughout endodontic treatment.

The most widely used endodontic irrigant is sodium hypochlorite (NaOCl). The advantages of NaOCl include: antimicrobial action against both planktonic microorganisms and biofilms, organic tissue dissolution, it is inexpensive, and it helps lubricate the canal during instrumentation (69, 91, 92). These characteristics, particularly the antimicrobial and tissue dissolution activity, can be enhanced by increasing the concentration or raising the temperature of the solution being used (93-95). Several characteristics contribute to the antimicrobial action of NaOCl: at its basic pH of 11, it dissociates into primarily hypochlorous acid ($HClO^-$) and as such, disrupts several cellular functions including oxidative phosphorylation, DNA synthesis, and several activities associated with the cell membrane of microorganisms (69, 96, 97). While these properties have made NaOCl the "gold standard" of endodontic irrigants, it does possess several drawbacks as well. NaOCl is cytotoxic to periapical cells and the periodontal apparatus if extruded outside of the root canal, it does not kill all bacteria nor inactivate endotoxins, it has an unpleasant odor/taste, it does not remove inorganic debris, and it does not possess substantivity (12, 69).

In order to address some of the limitations seen with NaOCl, other irrigants are used during endodontic treatment. A solution of 2% chlorhexidine gluconate (CHX) has been shown to be bacteriostatic at low concentrations, bactericidal at high concentrations, and to possess substantivity against many microorganisms due to its cationic nature (23, 98). CHX has a pleasant taste and odor and is much less cytotoxic against periapical and periodontal cells compared to NaOCl, rendering its use applicable in clinical situations

such as wide-open apices and root perforations (23). Since neither NaOCl nor CHX are capable of removing inorganic debris or the "smear layer" from inside the root canal, the addition of a chelating agent is necessary during endodontic therapy. Introduced into the specialty in 1957 by Nygaard-Ostby, a liquid solution of 17% ethylenediaminetetraacetic acid (EDTA) was shown to soften root canal dentin and dissolve the inorganic smear layer (99). Its use as a final rinse prior to root canal filling has been shown to facilitate dentinal tubule penetration of endodontic sealer to help ensure a complete and adequate seal during obturation (90). Other irrigants occasionally used include sterile water or saline as well as isopropyl alcohol for various reasons and in different scenarios of endodontic therapy (90).

OBTURATION

The third and final phase necessary for successful endodontic treatment, as laid out by Dr. G.G. Stewart, is obturation of the root canal. The root canal filling materials should be biocompatible and non-toxic to periapical tissues as well as able to form a uniform, homogeneous, and dense fill confined to the root canal space (80). Research has shown cases obturated to within 0-1 mm short of the radiographic apex show the highest rates of clinical success (100). Voids in obturations, filling 2 mm or more short of the radiographic apex, or obturations extending beyond the radiographic apex have been shown to decrease endodontic success rates (101). Today, the primary obturation materials used include some form of gutta percha along with an endodontic sealer. The use of sealer along with gutta percha assists in obtaining a hermetic seal of the root canal system (102).

MICROORGANISMS

Endodontic pathology results from interactions between microbes and the host's immune response. Ever since the classic study by Kakehashi, Stanley, and Fitzgerald, bacteria have been known to be the cause for endodontic disease and the associated pathology (1). Numerous studies have shown how bacteria can differ depending on symptoms, whether the endodontic infection is primary or secondary, or if it is an acute or chronic infection (103-105). Regardless of the specific microbes present, research has shown that they are typically arranged in a biofilm (106, 107). Biofilms are defined as microbial-derived, sessile communities characterized by cells irreversibly attached to a substratum or interface or to one another, embedded in a self-produced matrix of extracellular polymeric substances, and exhibiting an altered phenotype with respect to growth rate and gene transcription compared with their planktonic counterparts (107).

Bacteria organized in biofilms have been found to have certain mechanisms that bolster their resistance to environmental stresses and increase their rate of survival (108). It has been shown that biofilms can be up to 1,000-fold more resistant than their planktonic counterparts (36). Some of these qualities unique to biofilms include metabolic diversity, concentration gradients, genetic exchange, and quorum sensing (108). Biofilms are metabolically diverse, and this allows a sharing of nutritional resources and waste products resulting in greater overall survival. The concentration gradient created by the mere density of the biofilm allows for greater physical and chemical resistance to antimicrobials and the host's immune responses (108). Genetic exchange by the microbes in close contact allows for sharing of virulence factors.

Quorum sensing serves as a communication method among the microbial community and permits the members to act as a group and increase their overall effectiveness.

Endodontic infections are comprised of frequently isolated species that include both facultative and obligate anaerobes, including members of the *Streptococcus, Enterococcus, Prevotella, and Porphyromonas* species (109). Primary endodontic infections consist of an equal mix of gram-positive and gram-negative bacteria and contain mostly obligate anaerobes (103). Some examples include *Actinomyces naeslundii, Fusobacterium nucleatum*, and *Porphyromonas gingivalis* (110). Secondary infections differ from their primary counterparts in that they have been found to contain mostly gram-positive bacteria with a more equal distribution of facultative and obligate anaerobes present (104). *Enterococcus faecalis* is a gram-positive facultative anaerobe frequently found to be associated with secondary endodontic infections (111). Some of its virulence factors include its ability to adhere to dentin and invade dentinal tubules, to suppress lymphocytic action, to use serum for nutrition, and the possession of a proton pump which lowers the surrounding pH and increases resistance to calcium hydroxide (112, 113).

ENDODONTIC MANAGEMENT OF IMMATURE TEETH

One obstacle encountered in endodontic therapy involves treating immature teeth with incompletely formed roots and open apices. In addition to the obvious behavioral complications involved with treating younger patients, other, more technical challenges involved in these cases include difficulty fully instrumenting and disinfecting large, wide canal spaces (114), thin root walls susceptible to fracture (115), and open root apices with

no natural apical stop facilitating extrusion of chemical irrigants and obturating materials used during root canal therapy into the periapical tissues (116, 117). In order to overcome these challenges, various techniques have been developed over the years to help successfully treat these cases.

VITAL PULP THERAPY

Maintaining pulpal vitality, especially in permanent teeth with open apices, is the treatment modality of choice as it promotes continued root development and apical formation (118). Clinically, vital pulp therapy, often referred to as apexogenesis, includes pulp capping as well as partial or full pulpotomies. Each of these procedures differ in the amount of pulp tissue removal required. Factors that dictate how much tissue should be removed include size of pulpal exposure, amount of time passed since exposure, and ability to obtain hemorrhage control (119). According to the AAE glossary of terms, pulp capping involves "treatment of the exposed vital pulp by sealing with a dental material to facilitate the formation of reparative dentin and maintenance of the vital pulp." Pulp capping is indicated in cases with small carious or traumatic pulpal exposures with minimal hemorrhage (120). Pulpotomy treatment involves the removal of the coronal portion of a vital pulp to preserve the vitality of the radicular pulp and can be either complete or partial, depending on the extent of pulpal inflammation and ability to control bleeding (121).

Materials used in vital pulp therapy have evolved over the years. Historically, calcium hydroxide was the material of choice due to its disinfecting characteristics and ability to stimulate hard tissue deposition (122, 123). However, research has shown

several drawbacks to calcium hydroxide's use in vital pulp therapy. The hard tissue deposition initiated by calcium hydroxide takes months to form and is usually incomplete (124). In addition, due to its high pH, calcium hydroxide has also been shown to cause pulpal inflammation (125).

Due to the limitations of calcium hydroxide in vital pulp therapy, newer materials have been developed. Mineral trioxide aggregate (MTA) emerged in the field of endodontics as a more biocompatible pulp capping agent with superior hard tissue formation compared to calcium hydroxide (126, 127). MTA has found its niche in several endodontic clinical scenarios and has been used globally for many years. However, MTA has been found to be extremely difficult to handle, to have a long setting time, and to discolor teeth (128). To combat some of these limitations found with MTA, other bioceramic materials like Biodentine have been developed. Biodentine is a relatively new bioceramic material that exhibits excellent biocompatibility due to its similarity with biological materials like hydroxyapatite (129). Biodentine has been found to have excellent handling characteristics, sealing ability, and capability to regenerate damaged pulp and stimulate hard tissue formation (130). Vital pulp therapy relies on the maintenance of vital pulp tissue inside an immature tooth to ensure continued root development. In cases involving a necrotic, immature tooth with open apices, treatment options include apexification and regenerative endodontic procedures (REPs).

APEXIFICATION

Immature, permanent teeth with pulpal necrosis lack a sufficient apical barrier for obturation. The treatment aimed at creating such an apical barrier against which

endodontic filling materials can be compacted is referred to as apexification. The first apexification procedure involved chemomechanical debridement of the root canal followed by the placement of calcium hydroxide down into the root canal system and allowing a hard tissue barrier to form at the apex (131). Research showed that these barriers can take up to 12-24 months to form and that calcium hydroxide should be replenished every 3 months (116, 132). Though calcium hydroxide achieves relatively predictable results, long-term calcium hydroxide apexification has been associated with increased risk of cervical root fracture and poor patient compliance (133, 134).

To assuage some of the drawbacks seen with calcium hydroxide apexification, new techniques that implemented artificial apical barriers were proposed. MTA's biocompatibility and ability to seal in the presence of blood and other tissue fluids made it the natural choice of material for this situation (135, 136). The use of artificial barriers for apexification procedures significantly decreased treatment times for patients from several visits over multiple months to just one or two visits. While apexification may preserve an immature permanent tooth, this treatment is limited by its inability to induce continued root development or increase root thickness. As a result, these teeth have been shown to be more likely to experience root fractures compared to fully mature permanent teeth (137).

REGENERATIVE ENDODONTICS

Immature, permanent teeth with pulpal necrosis pose specific challenges to practitioners such as underdeveloped, thin root walls susceptible to fracture. As a result, alternatives to the apexification procedure have been explored for decades. Regenerative

endodontics refers to the "biologically based procedures designed to physiologically replace damaged tooth structures including dentin and root structures, as well as cells of the pulp-dentin complex." Nygaard-Østby introduced the idea to the world of endodontics in the 1960s when he showed tissue formation inside a root canal following pulp removal (138). However, regenerative endodontic procedures were relatively infrequent until Iwaya's case report using the "revascularization method" to treat an infected tooth with an immature root (139). Banchs and Trope successfully repeated Iwaya's work with a case report of their own that popularized the "revascularization method," the precursor to REPs (140). Three critical factors have been identified for REP success: stem cells, growth factors, and a scaffold (141). The scaffold and growth factors involved in REPs are believed to come from the dentin, fibrin clot, and sterilized pulp tissue remnants (142). The stem cells required are mesenchymal stem cells from the apical papilla (143).

Current AAE guidelines for REPs suggest treatment take place over two visits (144). At the first visit, following anesthesia and rubber dam isolation and after access into the necrotic pulp space, minimal instrumentation and gentle irrigation with 1.5% NaOCl and 17% EDTA is recommended (145, 146). Chlorhexidine and higher concentrations of NaOCl have been found to be toxic to the stem cells required for REPs and their use should be avoided in these cases (17, 147). After gentle instrumentation and irrigation, application of an intracanal medicament like triple antibiotic paste (7), double antibiotic paste (148), or calcium hydroxide (149) is indicated for 2 to 4 weeks (150). If the patient returns asymptomatic for the second visit, anesthesia without vasoconstrictor is administered to help preserve blood flow in the area. The root canal

system is rinsed with 17% EDTA to remove the smear layer and promote the release of growth factors from the underlying dentin (151). Bleeding into the root canal is then induced by over-instrumentation into the apical papilla and allowing blood to flow into the root canal system carrying with it the necessary stem cells and scaffold for REPs (140). Ideally, the blood should fill the canal just up to the cemento-enamel junction (CEJ) where a collagen barrier is placed followed by a bioceramic base and a permanent restoration (152). The main goals for REPs are 1) the elimination of symptoms and evidence of bony healing, 2) increased root wall thickness and/or increased root length, and 3) positive response to vitality testing (144).

USE OF OZONE FOR DISINFECTION

Ozone is a naturally occurring molecule consisting of three oxygen atoms and is most widely known for its role in the earth's atmosphere. The history of ozone began with its discovery in 1785 when Martinus Van Marum subjected oxygen atoms to electrical discharges creating "an odor of electrical matter" as well as an accelerated ability to oxygenate mercury (153). In 1840, these experiments were repeated by Christian Schonbein who concluded the odor was due to a gas that he termed "ozone," from the Greek *ozein*, meaning odorant, and described several of its properties (154).

Since that time, many researchers have worked tirelessly to understand the full nature and actions of ozone. Kleinmann conducted the first bacteriological studies with ozone shortly after its discovery (155). Its ability to destroy toxic or noxious industrial impurities and to inactivate bacterial contaminants made it a popular alternative to

chlorination. In 1901, Wiesbaden, Germany and Nice, France, became the first cities to utilize ozone to purify their drinking water.

Over the past century, ozone has been found to possess unique properties that make it applicable to certain biological systems as well as in both medical and dental clinical practice (156). In 1915, Wolff was credited with using ozone for treating infected wounds, ulcers, and osteomyelitis (157). During the First World War, ozone's bactericidal properties were recruited to treat wounds, mustard gas burns, and fistulas (158). However, these early attempts at using ozone for the treatment of wounds and certain medical conditions were met with several challenges.

Ozone is an extremely powerful oxidant, second only to fluorine in this regard. As a result, special materials must be used to store and administer ozone like glass, silicon, and Teflon as plastic will degrade over time as a result of ozone's oxidative power (28). While this property of ozone is crucial in its antimicrobial effectiveness, one must also consider its effect on human cells. Much research has been dedicated to this subject including its effect on the respiratory system if inhaled, on various cells throughout the human body, as well as its effect if injected directly into circulating blood. It has been well established that inhaled ozone can have both local and systemic repercussions. Humans exposed to ambient ozone may develop mildly accelerated breathing, irritation in the throat, and chest tightness (156). For this reason, during ozone therapy today, care is taken to avoid ozone's escape from the treatment area. Contemporary medical ozone generators convert ozone back into oxygen after it is administered. A high level of biocompatibility of aqueous ozone has been established against human epithelial cells, gingival fibroblasts, and periodontal cells (30-32). Topical

application of ozone is not toxic for human cells and has actually been shown to promote wound healing by increasing oxygen tension, increasing granulation tissue formation, and increasing the expression of transforming growth factor beta (TGF-B) and vascular endothelial growth factor (VEGF)(32). According to additional studies, the direct intravascular injection of ozone mixtures results in the following responses: 1) activation of enzymes involved in oxygen radical scavenging (e.g., catalase, superoxide dismutase), 2) acceleration of glycolysis in erythrocytes, resulting in 3) the stimulation of the 2,3 biphosphoglycerate cycle and a shift of the oxyhemoglobin curve releasing oxygen into tissues.

OZONE'S DIRECT MECHANISM OF ACTION

Ozone's bactericidal action is most commonly believed to be due to its disruption of the bacterial cell wall through its powerful oxidative capability. Bacteria contain an intricate cell wall composed of several different layers. The innermost layer is the cytoplasmic membrane made up of phospholipids and proteins. The outer layer consists of peptidoglycan molecules. In gram-positive bacteria, this peptidoglycan layer is thick and rigid while in gram-negative bacteria this layer is thin and contains lipoproteins and lipopolysaccharides. When ozone interacts with bacteria, it oxidizes the lipid and lipoprotein components of their cell wall. These "oxidative bursts" create holes in the cell wall and render the bacteria non-viable (28, 29, 156).

METHODS AND MATERIALS

HUMAN TOOTH SELECTION

Sixty (60) maxillary anterior human permanent teeth were collected and stored in 0.1% thymol solution. Teeth were visually inspected and any teeth with any of the following characteristics were excluded: less than 4mm midroot diameter buccolingually or mesiodistally, hypocalcification, restorations, decay, hypoplasia, fractures or cracks, incomplete root formation, fluorosis, dentinogenesis or amelogenesis imperfecta.

ROOT SPECIMEN PREPARATION

Tooth preparation and inoculation was completed using a protocol previously proven by Duarte *et al* (41). A diamond saw was used to cut off the crowns of all selected teeth. Root samples were prepared to a standardized length of 12mm. The root canals were then negotiated with a #10 endodontic hand file followed by a #15 hand file the full length of the root establishing apical patency. Working length was defined as 0.5mm short of apical patency length as viewed under a dental operating microscope. Once patent with a #15 hand file, the RCS was prepared with a WaveOne Gold White file (size 45.05) using a reciprocating motion in a Promark endodontic motor (Dentsply Sirona; York, PA) to the established working length. During preparation, the root canal was copiously irrigated with sterile saline. Following preparation, all canals were irrigated with 6% sodium hypochlorite for thirty seconds and then with 17% EDTA for thirty seconds to eliminate the smear layer. Once all root canals were prepared, specimens were stored in 0.1% thymol and then autoclaved for twenty minutes at 121° C prior to being used.

INOCULATION AND BIOFILM FORMATION

As described in previous studies, a standard strain of *E. faecalis* (ATCC 29212) was used to inoculate the prepared root canals in the following manner: a solution of brain-heart infusion broth (BHI) was inoculated with a single colony and incubated for 24 hours at 37°C at 5% CO_2 to form the stock culture. *E. faecalis* density for the stock culture was set to 3.2 x 10^7 CFU per milliliter. The teeth were then placed in 24-well culture plates (1 sample per well) filled with 1.8 mL of sterile BHI and 0.2 mL of fresh 24-hour stock inoculum and incubated at 37°C and 5% CO_2 for 14 days (159). BHI solution was replaced every 24 hours without the addition of new inoculum in order to prevent nutrient depletion.

PREPARATION OF OZONATED WATER

One hundred (100) mL of distilled water was bubbled in a glass upper column using a continuous flow of 12L/h of a mixture of 100% oxygen and ozone for 10 minutes (34). The ozone was generated using the Ozonobaric P (Sedecal, Medical) a portable ozone generator suitable for any kind of ozone therapy application and used to produce ozonated oil, water, or saline. Ozonated water was produced to the preset concentrations of 16 µg/mL, 25 µg/mL, and 50 µg/mL and immediately measured using an aqueous ozone monitor (UV-106-W) *following manufacturer's instructions*. The ozonated water was used immediately to prevent any significant decrease in ozone concentration.

EXPERIMENTAL GROUPS

After removal from the 24-well plates, specimens were divided randomly into six groups with 10 specimens in each, depending on the irrigant used (n=60). The experimental and control groups were as follows:

Group 1: 6% NaOCl irrigation (positive control)

Group 2: 1.5% NaOCl irrigation (recommended concentration for regenerative endodontic procedures)

Group 3: 16 µg/mL Ozonated water irrigation

Group 4: 25 µg/mL Ozonated water irrigation

Group 5: 50 µg/mL Ozonated water irrigation

Group 6: Saline irrigation (negative control)

Each specimen was irrigated with 5 mL of the corresponding irrigant via standard needle irrigation for 1 minute.

ASSESSMENT OF ANTIMICROBIAL ACTIVITY

After treatment, canals were gently flushed with sterile water to remove any residual irrigating solution within the root canal system. Next, 15 µL of sterile saline was injected into the RCS. The biofilm suspension was then sampled using sterile paper points for the apical third and a spiral utility brush (Versa Brush, Vista Dental, USA) in a slow-speed hand piece at 500 rpm for 1 minute for the coronal and middle thirds.

The paper points and spiral brushes were transferred to sterile tubes containing 5 mL of sterile saline. Biofilms were detached by sonicating for 20 seconds followed by vortexing for 30 seconds. A ten-fold serial dilution was completed, followed by plating

onto blood agar plates. *After incubation* for 48 hours in 5% CO_2 at 37°C, colonies were counted, and CFU/mL determined for statistical analysis

CONFOCAL IMAGING

Two teeth per group were prepared and treated as described above but prior to sterilization, the teeth were scored longitudinally as described in a previous study (160) in order to be analyzed with confocal imaging. This scoring allowed separation of the specimen with a scalpel after completion of treatment, exposing the root canal space for imaging. The canal spaces of all scored teeth were stained with Live/Dead® Bacterial Viability Kit (Baclight Bacterial Viability kit L7012; Molecular Probes, Inc). Confocal imaging allowed us to see both live and dead bacterial cells in all levels of the root canal space (coronal, middle, and apical thirds).

STATISTICAL ANALYSIS

The effect of treatment group on bacteria counts was made using one-way ANOVA. Pair-wise comparisons between the groups were made using the Sidak method to control the overall significance level at 5%. The distribution of the data was examined and a transformation of the data (e.g. $\log_{10}$) or nonparametric tests were used if necessary. A 5% significance level was used.

SAMPLE SIZE

Based on previous studies (25, 26) the coefficient of variation was estimated to be 0.75. With a sample size of 10 samples per group, the study was to have 80% power to detect a 3.5x difference between groups.

RESULTS

CFU counts were converted to $\log_{10}$ and compared using Fisher's Exact tests or one-way ANOVA followed by pair-wise tests. In all observations utilizing NaOCl irrigation, no colonies formed following treatment. CFUs were counted for the saline and ozone groups (Table I). The two NaOCl groups, with 0 CFU/mL, were significantly different than the other four groups (p=0.009). Saline showed a trend towards higher CFU/mL than 50 µg/mL O_3 (p=0.068). None of the other comparisons approached statistical significance (p=0.453 25 µg/mL O_3, p=0.606 16 µg/mL O_3, p=0.999 25 µg/mL O_3 vs 50 µg/mL O_3, p=0.990 16 µg/mL O_3 vs 50 µg/mL O_3, p=1.000 16 µg/mL O_3 vs 25 µg/mL O_3).

For confocal imaging, one full-length image of a sectioned root-half is included per group (Figures 12-16). Live cells fluoresce bright green whereas damaged or dead cells fluoresce bright red, which is a product of the solutions used for staining and imaging. The samples were stained with SYTO®9 and propidium iodide. These compounds both have a high affinity for nucleic acid (161). SYTO®9 is a smaller molecule with minimal charge, allowing it to cross the membrane of any cell, live or dead, and results in the bright green fluorescent color. Propidium iodide is a much larger molecule with a strong positive charge, preventing it from crossing the intact membrane of any live cells, but allowing it to bypass the damaged membranes of dead cells and results in the fluorescent red color. Propidium iodide has a much higher affinity for nucleic acid compared to SYTO®9 and can therefore displace the SYTO®9 molecules on any damaged/dead cells. The staining process involves multiple washes of saline and staining solution which could inadvertently displace some cells from the root surface and result in what appears to be black space. When NaOCl was used for irrigation, regardless

of the concentration, confocal imaging showed maximal killing of virtually all cells contained within the entire root canal space illustrated by the predominantly red fluorescent staining. Saline irrigation showed minimal killing throughout the root canal space with a full-thickness biofilm consisting of mostly live cells in the apical third. Irrigation with 16 µg/mL and 25µg/mL ozonated water showed a mixture of red and green cells in the coronal and middle thirds but a largely intact biofilm of live cells in the apical third or the root canal. Irrigation with 50 µg/mL ozonated water showed both live and dead cells throughout the entire length of the root canal space with more dead cells concentrated in the apical third compared to the other ozonated water groups and the saline group.

COVID-19 DISCLAIMER

The study was stopped early due to the COVID-19 closures with only n=7 per group instead of the planned n=10 per group. The pre-study power calculations indicated the study would have 80% power to detect a 3.5x difference in means with n=10 per group and 5 study groups. Only the comparison between Saline and 50 ug/ml O_3 reached the 3.5x difference and approached statistical significance (p=0.068). The increase in the number of groups and the decrease in the sample size both affected the power of the study – 43% power to detect a 3.5x difference, or 80% power to detect a 5.7x difference.

STATISTICAL METHODS

Because all observations had 0 CFU/mL for the two NaOCl groups, comparisons of these two groups against the other four groups for CFU/mL > 0 or not were made

using Fisher's Exact tests. Comparisons among the other four groups were made using one-way ANOVA followed by pair-wise tests. The Sidak method was used to control the overall significance level the tests at 5%. All analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).

FIGURES AND TABLES

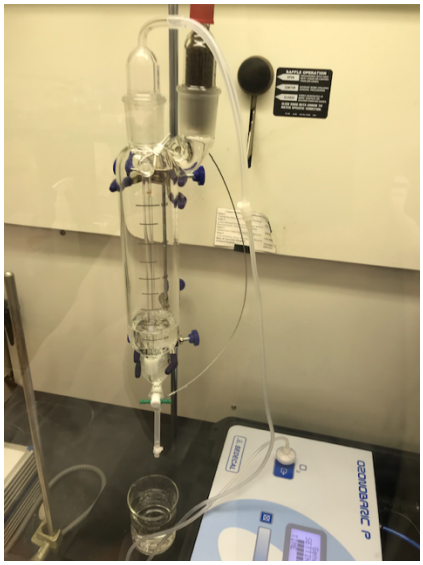


FIGURE 1. Sedecal Ozonobaric P Ozone Generator

FIGURE 2. Tooth samples of approximately similar size

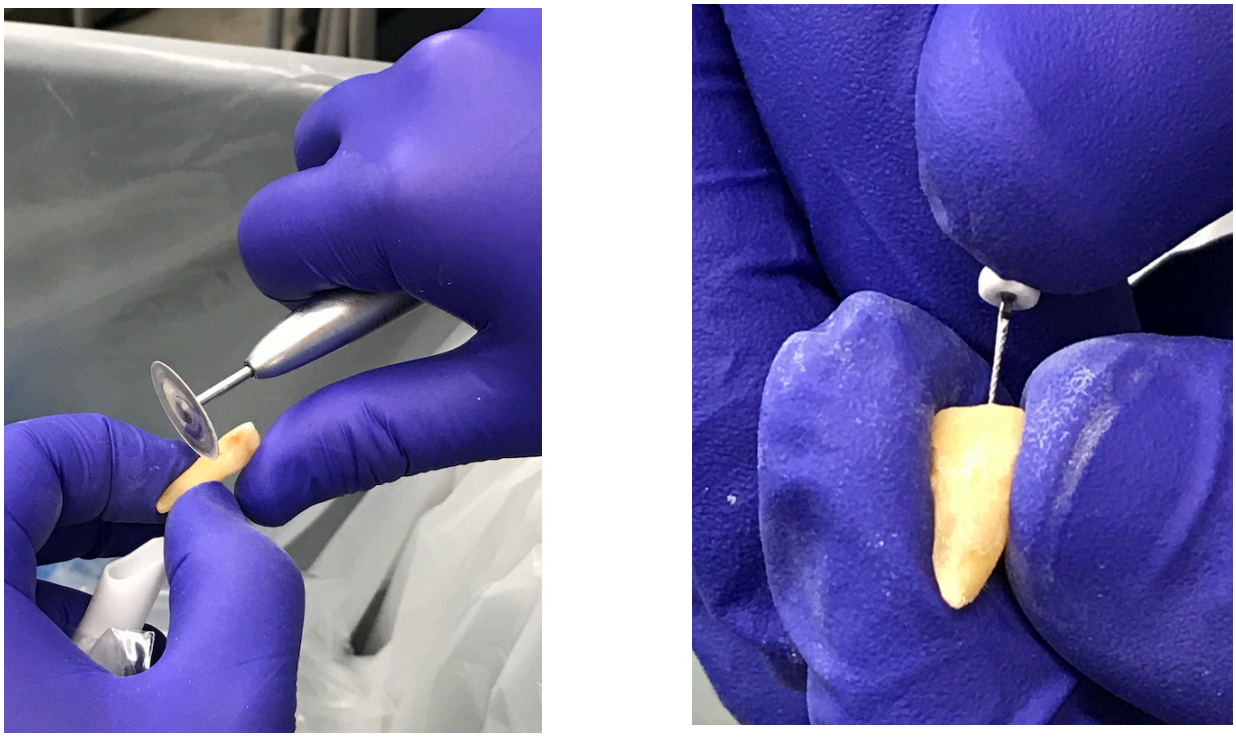


FIGURE 3. Decoronation to standard 12mm root length and canal preparation

FIGURE 4. Apical patency obtained with #10-K file

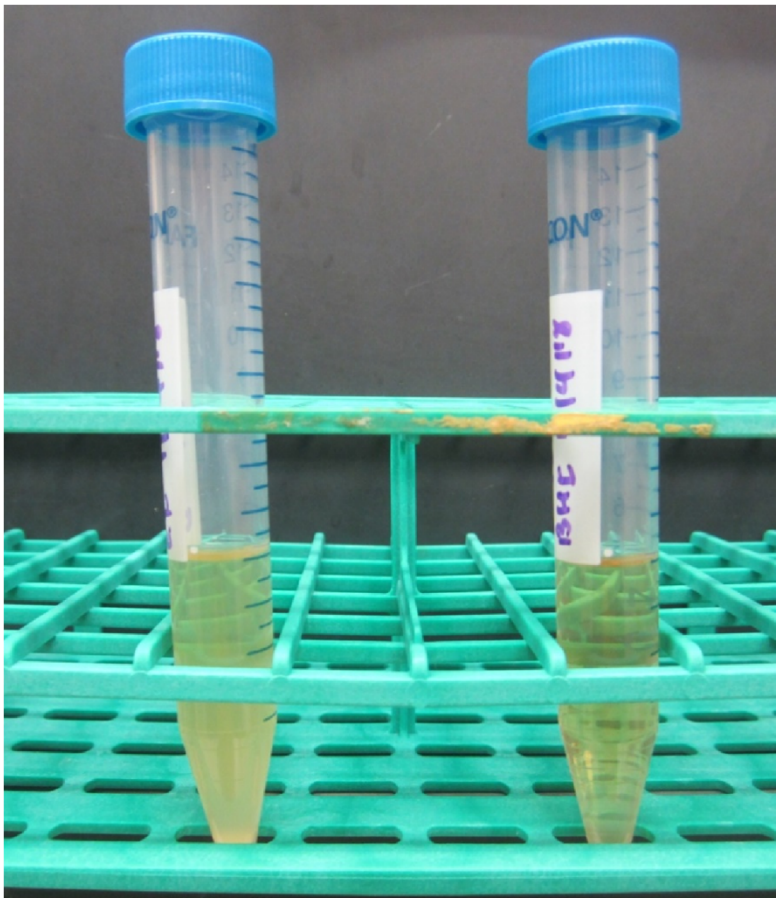


FIGURE 5. *E. faecais* stock inoculum (left) is cloudy compared with the sterile control (right)

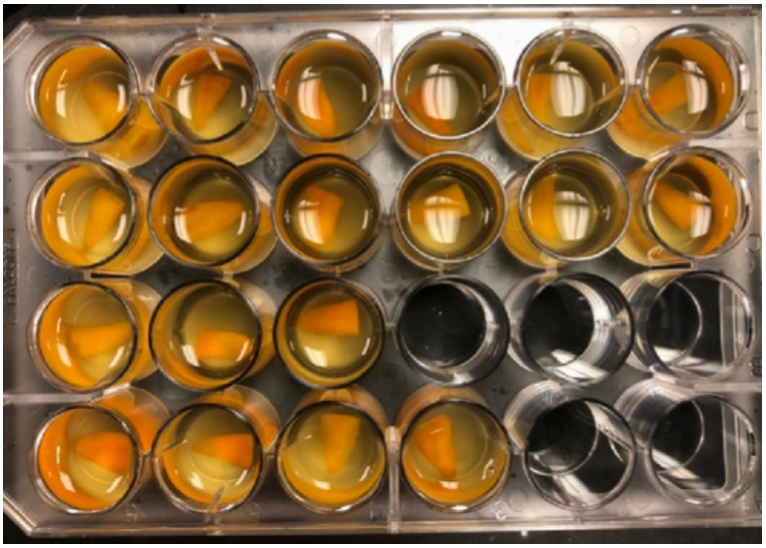


FIGURE 6: Inoculated specimens

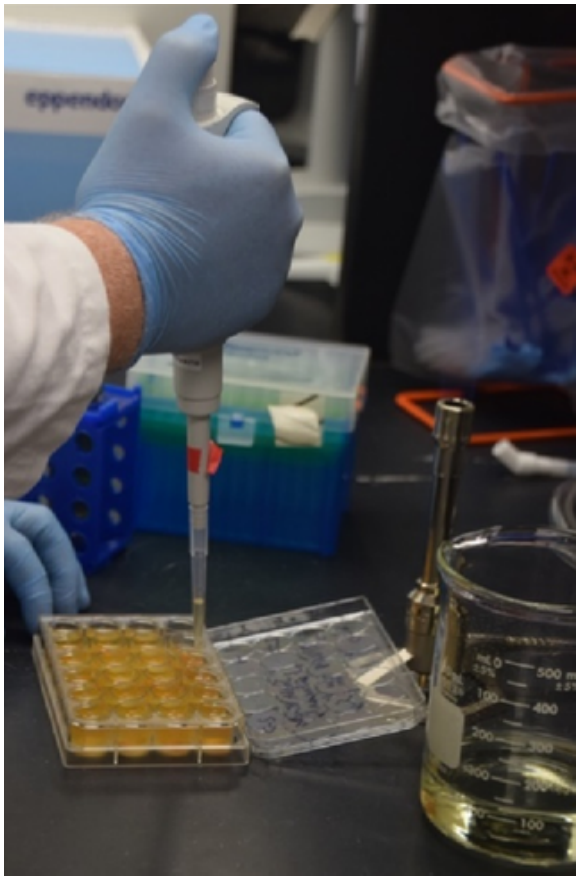
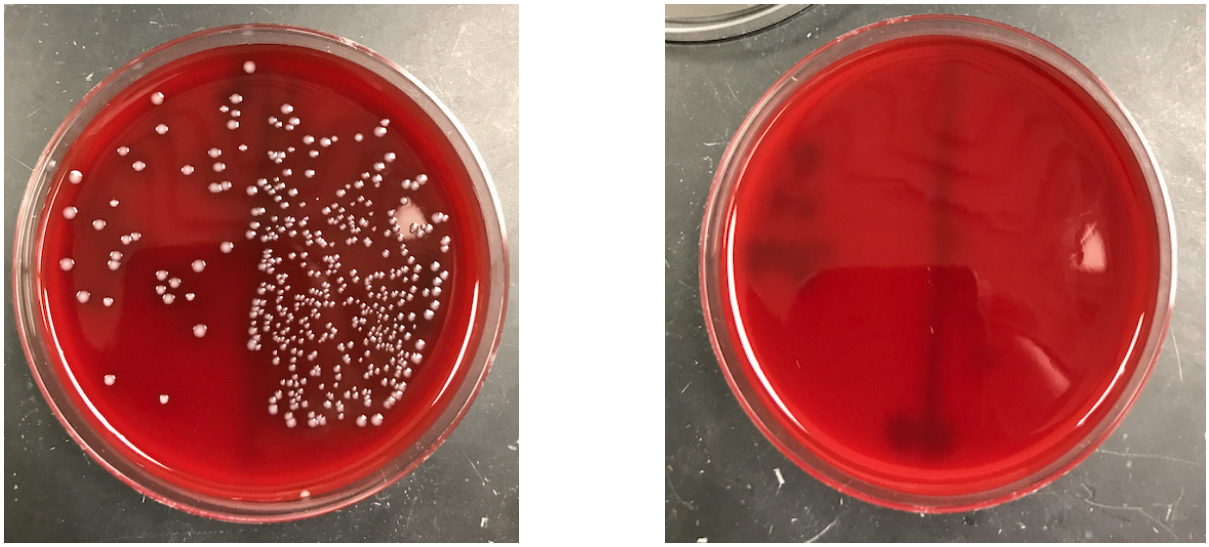


FIGURE 7. Changing BHI media



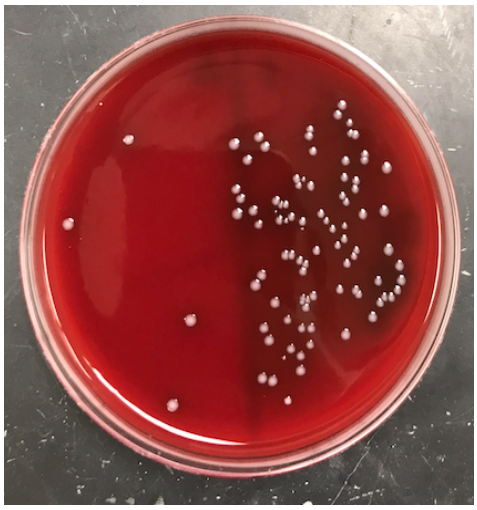
A. B.

FIGURE 8. Representative growth plates split in half by dilution:

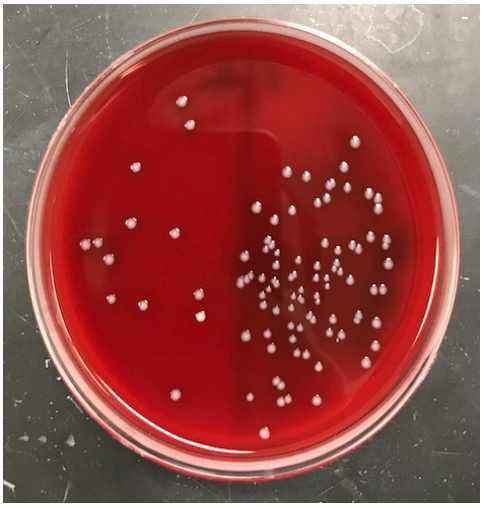
(A) Negative control – saline (10^{-1} and 10^{-2} dilutions);

(B) Positive control – 1.5% NaOCl (0 and 10^{-1} dilutions)

A.



B.



C.

FIGURE 8. Representative growth plates split in half by dilution:

(A) 16 µg/mL ozonated water; (10^{-1} and 10^{-2} dilutions);

(B) 25 µg/mL ozonated water; (10^{-1} and 10^{-2} dilutions);

(C) 50 µg/mL ozonated water; (10^{-1} and 10^{-2} dilutions)

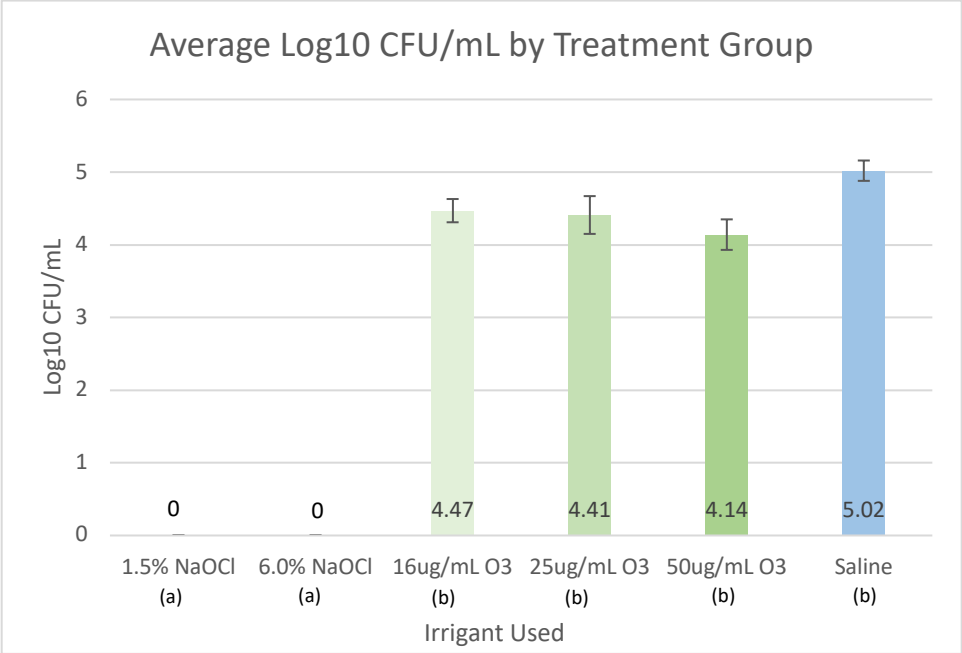


FIGURE 9. Average Log10 CFU/mL count per group (a different letter indicates a statistically significant difference from the other groups).

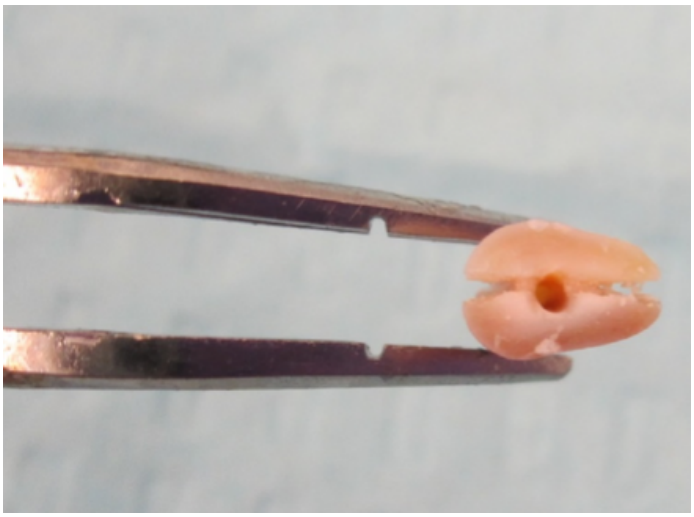


FIGURE 10. Scoring a tooth for confocal imaging

FIGURE 11. A scored and split tooth

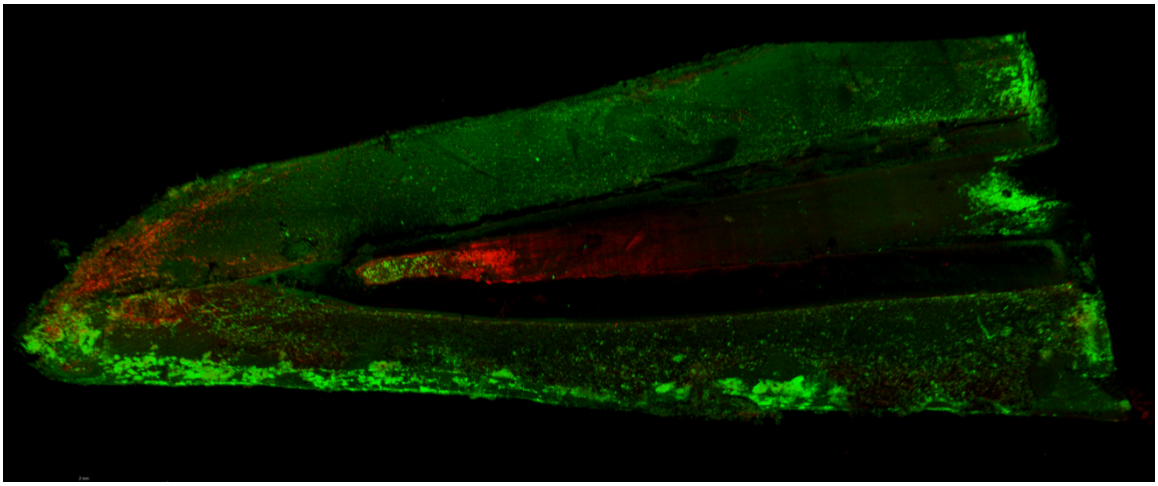


FIGURE 12. Confocal imaging of root canal irrigated with saline

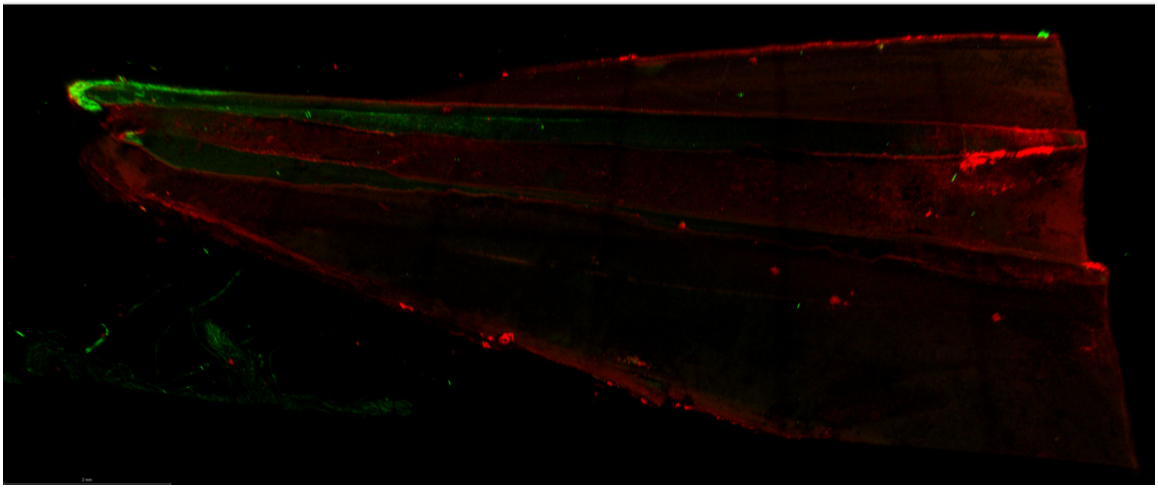


FIGURE 13. Confocal imaging of root canal irrigated with 1.5% NaOCl

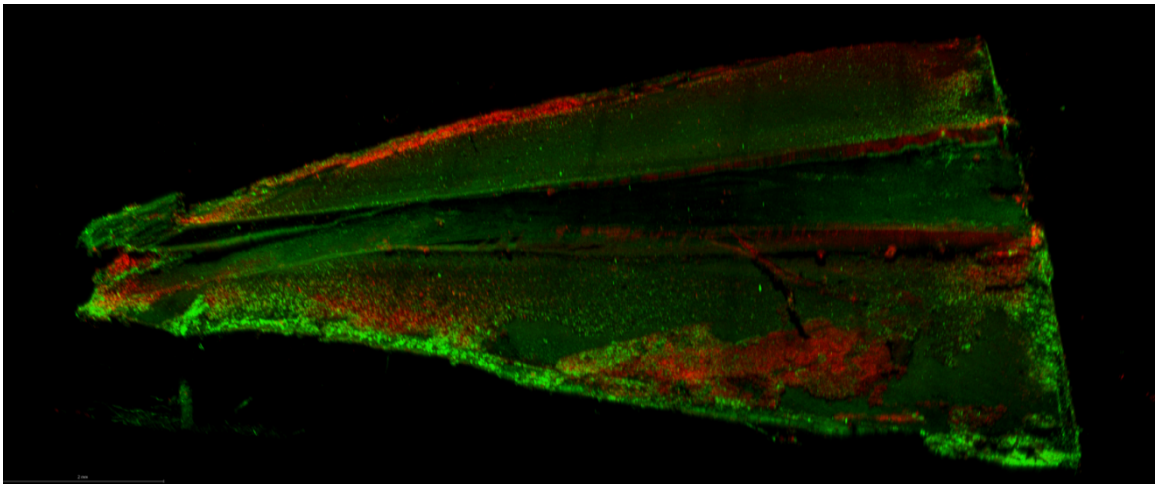


FIGURE 14. Confocal imaging of root canal irrigated with 16µg/mL ozonated water

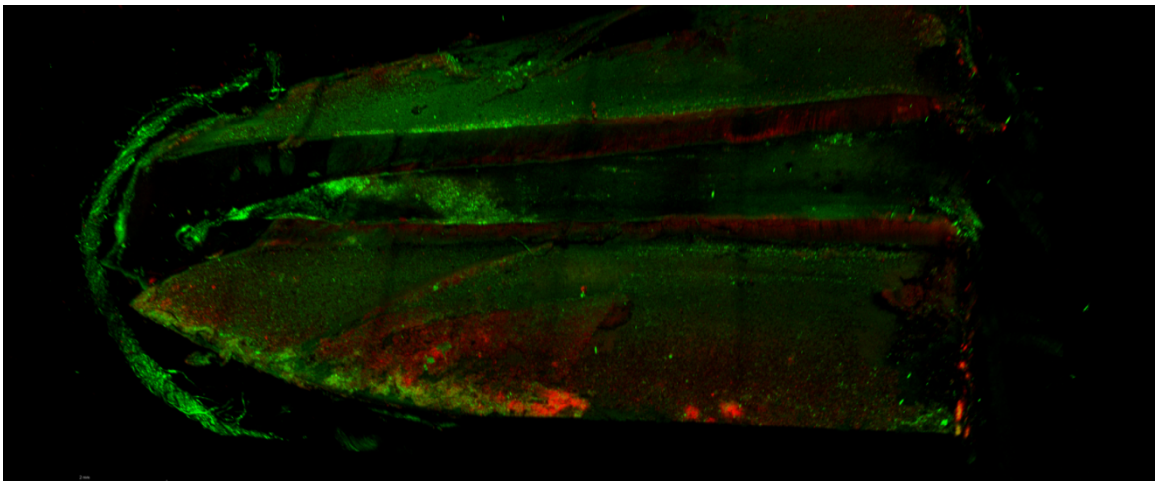


FIGURE 15. Confocal imaging of root canal irrigated with 25µg/mL ozonated water

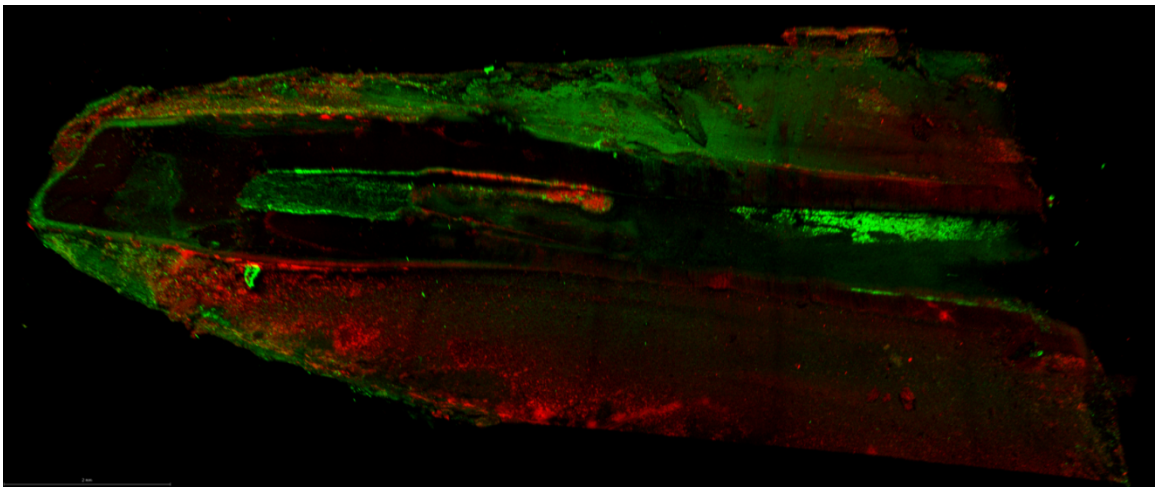


FIGURE 16. Confocal imaging of root canal irrigated with 50µg/mL ozonated water

		CFU/mL					Log_{10}(CFU/mL)		
Group	**N**	**Mean**	**SD**	**SE**	**Min**	**Max**	**Mean**	**SD**	**SE**
1.5% NaOCl	7	0	0	0	0	0	0.00	0.00	0.00
6% NaOCl	7	0	0	0	0	0	0.00	0.00	0.00
16 µg/mL O_3	7	45314	49701	18785	11800	146000	4.47	0.42	0.16
25 µg/mL O_3	7	64743	86985	32877	3600	242000	4.41	0.70	0.26
50 µg/mL O_3	7	20294	11781	4453	860	33800	4.14	0.55	0.21
Saline	7	134086	80147	30293	23600	228000	5.02	0.37	0.14

Table I
Log_{10} CFU/mL counts as a function of treatment rendered

DISCUSSION

It is well-known and accepted that chemical irrigation in root canal therapy serves a critical role in microbial reduction of the infected root canal space and is paramount to the success of endodontic treatment (90-93). Peters et al. showed that mechanical instrumentation alone left at least 35% of root canal walls untouched, substantiating the importance of chemical irrigation to reach and disinfect these uninstrumented areas (8). Globally, the "gold standard" endodontic irrigant is NaOCl, of various concentrations, due to its powerful antimicrobial capacity, its ability to dissolve organic tissues, and its availability and low cost to use. However, NaOCl does not come without several drawbacks. At certain concentrations, it has been shown to be cytotoxic to periapical cells and cells of the periodontal attachment apparatus, particularly stem cells of the apical papilla (SCAPs) necessary for regenerative endodontic procedures, it also has a foul odor/taste and it can have devastating consequences if extruded beyond the apical foramen of teeth resulting in intense pain, bruising, and swelling and in some cases can potentially lead to paresthesia, secondary infections, and even life-threatening complications (17, 162). For these reasons, much research has been dedicated to developing an alternative endodontic irrigating solution to address some of the pitfalls of other current irrigating solutions and to facilitate treatment in various endodontic procedures.

Previous research involving ozone's antimicrobial effect in endodontic treatment has involved various forms of ozone at different concentrations and has primarily evaluated its effect on planktonic bacteria (24-26, 30). Research is lacking regarding the use of ozonated water as an endodontic irrigant and its ability to disrupt an established, mature intracanal biofilm.

In our study, all samples treated with any concentration of NaOCl showed a statistically significant difference in CFU/mL compared to all other treatment groups evidenced by no colonies detected in any NaOCl culture sample. This finding corroborates the results of previous studies that have shown complete eradication of an *E. faecalis* biofilm with as low as 0.00625% NaOCl (163, 164). The specific concentrations of 6.0% and 1.5% NaOCl chosen for this experiment were based on the current irrigants used in most traditional nonsurgical root canals completed in the United States and the AAE's guidelines for regenerative endodontic procedures today, respectively. Future studies involving ozonated water irrigation could consider using lower concentrations of NaOCl than were used in this study. However, since 1.5% is often the lowest concentration used in endodontic procedures today, with research to back its safety and efficacy (17, 163), results may not be clinically relevant.

Based on the results of our study, standard-needle irrigation with ozonated water at a concentration of at least 50µg/mL is capable of elucidating an anti-biofilm effect against a 2-week-old biofilm of *E. faecalis* compared to irrigation with saline. There was a trend towards a lower number of CFU/mL between the 50µg/mL ozonated water group and the negative control saline group ($p=0.068$). In addition, confocal imaging of the roots irrigated with 50µg/mL ozonated water helped illustrate this effect by showing a larger area of dead or missing cells in comparison to the roots irrigated with saline. As the concentration of ozonated water increased in this experiment there was an associated decrease in CFU/mL. Considering this trend that illustrates an antibiofilm effect in the presence of dentin, ozonated water could potentially serve a role in some areas of endodontic therapy; i.e., as an intracanal medicament in between appointments. Previous

studies have shown that dentin can buffer the effects of the more commonly used intracanal medicament calcium hydroxide (165). Furthermore, one advantage ozonated water could have over calcium hydroxide as an intracanal medicament is the fact that ozonated water could be completely removed from the canal system whereas calcium hydroxide cannot (166, 167). Future studies might use a similar protocol to investigate stronger concentrations of ozonated water against an established *E. faecalis* endodontic biofilm as well as compare those concentrations to calcium hydroxide against a similar biofilm.

Human teeth were used in this study as opposed to plastic wells because we believed this model would be more relevant and applicable to real life clinical scenarios. In addition, using the entire root, as opposed to dentin slices, allowed us to visualize the antibiofilm effects throughout the entire root length including the coronal, middle, and apical thirds. The purpose of scoring and sectioning the roots, followed by exposing the canals to confocal imaging was to provide an illustration of the effects the various irrigants had on an intact, non-disrupted bacterial biofilm throughout the entire length of the canal. Previous studies have used intracanal dentinal chips obtained after treatment with burs or files or prepared root sections to evaluate the post-treatment biofilm. While these techniques may offer some valuable deductions, we believed scoring the roots prior to treatment and sectioning the roots after treatment would allow for the least amount of disruption to the intracanal biofilm and provide the most clinically relevant illustration of how different irrigating solutions worked throughout the entire root canal space. The black areas seen throughout the root canal spaces in the confocal images obtained might be explained as a consequence of the irrigation method used. Standard-needle irrigation

with the same volume of solution over the same amount of time was used for every sample. However, during standard-needle irrigation, shear forces are generated as the irrigant is expressed and the irrigation needle is moved up and down within the canal. These forces could have disrupted the biofilm resulting in the black areas seen in the confocal imaging. To address this potential issue, future studies may utilize different techniques such as using contact time instead of passive irrigation to measure irrigant anti-biofilm efficacy. For example, to avoid creating these undesirable forces, one could fill the root canal space with the irrigant and allow it to set for a standard amount of time before antimicrobial analysis.

There was no statistically significant difference in CFU/mL between the 16µg/mL and 25µg/mL ozonated water groups compared to saline. Hubbezoglu et al. evaluated aqueous ozone's effects on an *E. Faecalis* biofilm at concentrations of 8µg/mL, 12µg/mL, and 16µg/mL and showed effective elimination of the biofilm with aqueous ozone at a concentration of 16µg/mL (26). This previous study helped establish the concentrations of ozonated water to be used in our study. Several explanations may exist for the discrepancy in results between the two studies. First, the *E. faecalis* biofilm used by Hubbezoglu et al. was only allowed to mature for 24 hours compared to the 2 weeks allotted in the present study. Second, ultrasonic application was used with aqueous ozone irrigation for 180 seconds in their study whereas only standard-needle irrigation for 60 seconds was used in our study. Previous research has shown that ultrasonic activation of endodontic irrigants results in improved canal cleanliness, better irrigant transfer throughout the root canal system, soft tissue debridement, and removal of the smear layer and bacteria (168). In addition, it has been shown that the more established and mature a

biofilm is, the more difficult it is to completely eradicate (164). Future studies investigating ozone as a potential endodontic irrigant should standardize protocols in order to control as many variables as possible.

Our study used a single-species biofilm of *E. faecalis* that was grown for 2 weeks. While this allowed us to reduce the number of variables involved and created a simpler experimental design, it is in contrast to true *in vivo* endodontic infections which are polymicrobial in nature. Compared to single-species biofilms, polymicrobial biofilms are able to work synergistically, express different virulence factors, and better evade antimicrobial action making them much harder to eliminate (169). Future studies should evaluate ozonated water irrigation against established, polymicrobial biofilms.

Previous studies have shown a high level of biocompatibility of aqueous ozone on human epithelial cells, gingival fibroblasts, and periodontal cells as well as its ability to promote wound healing (31, 32). However, to the best of our knowledge, evidence-based studies are lacking concerning aqueous ozone's effect on stem cells, specifically stem cells of the apical papilla involved in the ever-evolving field of regenerative endodontics. Irrigants used in regenerative endodontic procedures need to be both antimicrobial, to eliminate the infection, and non-toxic, to ensure the viability of the apical stem cells. Trevino et al. showed the effects of various root canal irrigants on the survivability of human stem cells of the apical papilla *in vitro* (147). A potential promising area of future research could use a similar protocol and evaluate various concentrations and forms of ozone on the viability of these stem cells for possible future use in regenerative endodontic procedures.

SUMMARY AND CONCLUSIONS

The results of this study failed to reject the null hypothesis. There was a statistically significant difference in the *E. faecalis* biofilm remaining in the groups treated with ozonated water compared to those treated with NaOCl. However, there was a trend towards higher CFU/mL in the saline group compared to the 50µg/mL ozonated water group. According to this finding, future studies should evaluate the effects of higher concentrations of ozonated water against an established *E. faecalis* biofilm. In addition, other follow-up studies might include ozonated water's effect on human cells, such as the stem cells of the apical papilla that are so critical to the success of regenerative endodontic procedures.

Due to university and laboratory closures caused by the COVID-19 pandemic, this project was stopped short and an insufficient sample size did not allow for proper statistical power. Additional occasions should be run upon the university's re-opening to allow for proper statistical power.

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ABSTRACT

EFFECTIVENESS OF OZONATED WATER IRRIGATION AGAINST AN ESTABLISHED *ENTEROCOCCUS FAECALIS* BIOFILM IN ROOT CANAL TREATED TEETH *IN VITRO*

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Introduction*:* One of the main objectives of endodontic therapy is to reduce microbes and remove inflamed pulpal tissue within the root canal system (RCS). This is accomplished through chemomechanical debridement of the RCS using hand and rotary instrumentation along with an antimicrobial irrigant. Today, the most commonly used irrigant is sodium hypochlorite (NaOCl), often at concentrations toxic to human cells. The use of ozone as an endodontic irrigant is a novel technique that has been proven to be antimicrobial against several microorganisms. However, independent research is lacking on ozone's efficacy against an established endodontic biofilm. If ozone's efficacy against biofilms is confirmed, the use of toxic and potentially dangerous sodium hypochlorite could be replaced in some clinical situations (i.e., regeneration, immature teeth, resorption) with a safer and effective alternative.

Objective: The aim of the current study was to evaluate the anti-biofilm activity of different concentrations of ozonated water compared to various concentrations of NaOCl against an established endodontic biofilm of Enterococcus *faecalis* in root canal treated teeth *in vitro*.

Materials and Methods: The crowns of similarly sized, maxillary anterior teeth were removed, and the roots cut to a standard length (12 mm). All root canals were instrumented to a standard size. Specimens were sterilized and then inoculated with *E. faecalis*, which were allowed to grow for two weeks to form an established biofilm. There were six treatment groups: 1) 6% NaOCl; 2) 1.5% NaOCl; 3) 16µg/mL ozonated water; 4) 25µg/mL ozonated water; 5) 50µg/mL ozonated water, and 6) saline. Following treatment, samples were collected, plated, and incubated for two days. The number of CFU/mL were determined, and samples visualized using confocal imaging.

The effect of treatment group on bacterial counts was made using one-way ANOVA followed by pair-wise comparisons.

Null Hypothesis: Endodontically treated teeth irrigated with ozonated water will not demonstrate a statistically significant decrease in the *E. faecalis* biofilm compared to those treated with sodium hypochlorite

Results: CFUs were converted to $\log_{10}$ and compared using Fisher's Exact tests or one-way ANOVA followed by pair-wise tests. In all observations utilizing NaOCl irrigation, no colonies formed following treatment. The two NaOCl groups, with 0 CFU/mL, were significantly different than the other four groups (p=0.009). Saline showed a trend towards higher CFU/mL than 50 µg/ml O_3 (p=0.068). None of the other comparisons approached statistical significance (p=0.453 25 µg/ml O_3, p=0.606 16 µg/ml O_3, p=0.999 25 µg/ml O_3 vs 50 µg/ml O_3, p=0.990 16 µg/ml O_3 vs 50 µg/ml O_3, p=1.000 16 µg/ml O_3 vs 25 µg/ml O_3). Confocal imaging helped illustrate effects of irrigation and confirm CFU findings.

Conclusion: The results of this study failed to reject the null hypothesis. There was a statistically significant difference in the *E. faecalis* biofilm remaining in the groups treated with ozonated water compared to those treated with NaOCl. However, there was a trend towards higher CFU/mL in the saline group compared to the 50µg/mL ozonated water group. According to this finding, future studies should evaluate the effects of higher concentrations of ozonated water against an established *E. faecalis* biofilm. In addition, other follow-up studies might include ozonated water's effect on human cells, such as the stem cells of the apical papilla that are so critical to the success of regenerative endodontic procedures.

Due to university and laboratory closures caused by the COVID-19 pandemic, this project was stopped short and an insufficient sample size did not allow for proper statistical power. Additional occasions should be run upon the university's re-opening to allow for proper statistical power.

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